Relationships between hexapods and crustaceans based on mitochondrial genomics

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ABSTRACT

The ever-increasing use of molecular data in phylogenetic studies have revolutionized our view of the phylogenetic relationships among the major lineages of arthropods. In this context, an important contribution is offered by mitochondrial genes, and the now widely available sequences of entire mitochondrial genomes. One of the most debated issues in arthropod phylogeny is the relationships between crustaceans and hexapods, and particularly whether the traditional taxa Crustacea and Hexapoda are mono- or paraphyletic. A key role is played by basal hexapodan taxa, the entognathan apterygotans (Protura, Collembola, Diplura), whose phylogenetic position as the sister-taxa of the Insecta *s.s.* is not totally convincing. The phylogenetic analysis based on mitochondrial protein-coding genes suggests that there are crustacean taxa which are more closely related to the Insecta *s.s.* than are Collembola and Diplura, therefore suggesting non-monophyly of the taxon Hexapoda as traditionally defined. Hence, Collembola and Diplura might have differentiated from their pancrustacean ancestor(s) before the remaining hexapods (Insecta) differentiated fomr their closest relative with crustaceans. These results also imply a new scenario for the evolution of several morphological and physiological features of hexapods, including terrestrialization.

1 INTRODUCTION

In the last 10 years, mitochondrial genomics (the analysis of sequence and structural features of the mitochondrial genome) has had a considerable impact on the reconstruction of higher-level phylogeny among Arthropods. For example, Boore et al. (1995), and, more clearly, Boore et al. (1998) showed that the translocation of a tRNA gene links crustaceans and hexapods, to the exclusion of myriapods (and others), contributing to the widely accepted dismissal of the Atelocerata concept (Telford & Thomas 1995), in favour of the Pancrustacea (=Tetraconata; Dohle 2001). Other studies using both molecular (Friedrich & Tautz 1995; Regier & Shultz 1997) and developmental (Averof & Akam 1995; Panganiban et al. 1995) data have supported similar relationships, although in some cases this aspect was not emphasized. Subsequently, a considerable bulk of data have been produced in support of the Pancrustacea hypothesis, owing to renewed interest and the collection of new phylogenetic evidence from different perspectives: developmental genetics (Cook et al. 2001; Deutsch 2001), neurobiology (Duman-Scheel & Patel 1999; Dohle 2001; Simpson 2001), skeletal structures (Deuve 2001), and the sequences of nuclear genes (Shultz & Regier 2000; Regier & Shultz 2001). Mitochondrial genomics has continued to contribute

extensively to phylogenetic studies given the signal that could be recovered from the nucleotide and the putative amino acid sequences of some, or all, mitochondrially-encoded protein coding genes (Garcia-Machado et al. 1999; Wilson et al. 2000; Nardi et al. 2001; Lavrov et al. 2004; Negrisolo et al. 2004). The considerable amount of molecular and developmental data supporting the Pancrustacea is in striking contrast with what had been apparently quite convincing morphological evidence supporting a Myriapoda-Hexapoda relationship, some of it even collected in recent times (Koch 2001; Kraus 2001). However, it now seems that the Pancrustacea concept is favored over that of the Atelocerata by most systematists.

Considering the recently developed view that Crustacea may be paraphyletic, most efforts are now placed on the identification on which crustacean lineage should be considered the sister taxon of the Hexapoda (Schram & Jenner 2001; Lavrov et al. 2004). In this quest, a crucial role is played by the most basal lineages of six-legged arthropods, which comprise five major taxa of quite neglected, soil-dwelling animals, collectively known as apterygotans: Protura, Collembola, Diplura, Microcoryphia and Zygentoma. According to the classical view, the first three of these taxa, which share entognathan mouthparts, branched off earlier along the hexapod lineage, before their closest relatives acquired ectognathan mouthparts and, later, the ability to fly. Due to the many peculiar features they possess, entognathan taxa are not usually granted the status of insects, and the taxon Insecta is formerly limited to the ectognathan orders (Kristensen 1981). While the monophyly of Hexapoda has hardly been questioned at all (Wheeler et al. 2001), considerable debate has grown over the phylogenetic relationships of the entognathan groups, either challenging the monophyly of the taxon Entognatha (Kukalová-Peck 1987; Kristensen 1997), or the monophyly of some of its taxa, such as the Diplura (Stys & Bilinski 1990). Although the monophyly of Hexapoda has been broadly accepted, a closer look to the pertinent literature shows that the number of shared features is very small (Bitsch & Bitsch 1998; Klass & Kristensen 2001), and therefore the support in favour of their monophyly arguably weak (Friedrich & Tautz 2001). This question merits further testing.

The difficulty in establishing phylogenetic relationships at this level arises from the fact that these splitting events have occurred long ago, in the Devonian (Whalley & Jarzembowsky 1981) or even the Silurian (Engel & Grimaldi 2004), and that these taxa might be derived from a sudden radiation that took place in a relatively short period of time. Again, molecular data have been important in stimulating the discussion on this subject by showing that the position of apterygotans, represented by collembolans in most molecular studies, as the sister-taxon of the Insecta was not really very well supported (Spears & Abele 1997; Shultz & Regier 2000). Further evidence was subsequently provided through the phylogenetic analysis of mitochondrial protein coding genes (Nardi et al. 2001, 2003; Negrisolo et al. 2004), which consistently shows that there are crustacean taxa which are more closely related to the Insecta than collembolans, and that these latter branched off the pancrustacean lineage before the diversification of the stem lineage of Insecta from their putative crustacean ancestor. This hypothesis implies the non-monophyly of the Hexapoda (as traditionally defined), and an alternative view of some basic steps of the evolution of arthropods, such as terrestrialization.

2 THE DATA

The complete sequence of the mitochondrial genome is available for quite a number of arthropod taxa, so we have concentrated our sequencing efforts in the apterygotan hexapods that have been generally neglected thus far. Currently, two sequences are available from Collembola: the onychiurid *Tetrodontophora bielanensis* (Nardi et al. 2001) and the hypogastrurid *Gomphiocephalus hodgsoni* (Nardi et al. 2003). Also available and of special interest is the sequence of the zygentoman *Tricholepidion gertschii* (Nardi et al. 2003), considered to be one of the most basal taxa of the Dicondylia (Pterygota + Zygentoma).

Adding to this, we recently determined the complete mtDNA sequence of a dipluran (the japygid *Japyx solifugus*), which will be described in more detail elsewhere. The sequence of the complete genome was obtained with a combination of primer walking and shotgun sequencing approach, based on the amplification of the whole molecule in long fragments. First, we amplified two long fragments encompassing the regions between the *cox1* and *cox3* genes (with the universal primers C1-J-1751 and C3-N-5460: Simon et al. 1994), and the region between the *cox3* and *nad4L* genes (with the primers 5'-CTCCCATAGGCATTTCACCATTCAA-3' and 5'-GCTTTCGGGGGGTGTGTGTGGTTATTT-3'). These two fragments were completely sequenced *via* primer walking. We also amplified and sequenced a small fragment encompassing *rrnL* and *rrnS* using the universal primers LR-J-13417 and SR-N-14588 (Simon et al. 1994). Then, we designed primers specific for *J. solifugus* using the known sequences of *cox1*, *nad4*, *trnV* and *rrnS* (respectively: 5'-AAAGCCCAGTGCTCACAGAATGGACG-3', 5'-GACCAATAACCATTCTACGACTACCAACACG-3', 5'-GAATTGCACAGATCCTACTCAGTGTA-3' and 5'-GGTGTGTACATATCGCCCGTCACTCTC-3'), and used these primers to amplify the remaining part of the genome in two long fragments (*trnV-cox1* and *nad4-rrnS*). This was achieved with a long-PCR approach producing two fragments of about 5.7 Kb and 4.2 Kb, respectively. The long-PCR products were then purified with Microcon PCR (Amicon-Millipore), and sheared, by running them through a Hydroshear (GeneMachines), into ~1.5 Kb fragments which were subsequently pooled together and cloned. Over 350 clones were sequenced from this library, and the sequences were automatically assembled using the software Sequencher. Due to the considerable number of clones sequenced, we obtained an average of 5-fold coverage on each position of the mtDNA sequence.

The mitochondrial genome of *Japyx solifugs*, 15,785 bp long, shows the same gene content typical of most metazoans (Boore 1999), and the same gene order as *Gomphiocephalus hodgsoni*, *Tricholepidion gertschii* and *Drosophila* (Clary & Wolstenholme 1985). This gene order, shared also with *Daphnia pulex* (Crease 1999) and differing by only the position of one tRNA gene from the mtDNA of *Limulus polyphemus* (Lavrov et al. 2000), is believed to be the ancestral arrangement of the Pancrustacea (Crease 1999). Interestingly, it differs from the gene order of one of the two collembolans, *Tetrodontophora bielanensis*, for two tRNA translocations (Nardi et al. 2001), which therefore are likely to be autapomorphic features of an internal lineage of Collembola. Hence, gene order does not provide useful information to reconstruct the phylogeny of these lineages of arthropods, nor to test whether hexapods are monophyletic.

3   THE ANALYSIS

Therefore, we turned our attention to comparisons of the sequences. Since changes in the nucleotide sequences are long saturated at this level of divergence, we focussed on the less-variable amino acid sequences. In order to perform a preliminary survey of the rates of variability and the levels of confidence in the alignment (i.e. the establishment of homology across positions), we aligned the amino acid sequences of all 13 mitochondrially-encoded proteins from all the species with complete mtDNA sequences available at the time of this analysis.

The alignment was performed using ClustalX (default settings; Thompson et al. 1997), followed by visual inspection. It was soon clear that some parts of the genome, and even some entire genes, were very difficult to align, given the exceptional levels of variability (both in primary sequence and length) across the range of selected taxa. Many aligned genes had less than 15% of invariable sites, with gaps introduced at over 25% of positions (Nardi et al. 2003). In order to minimize the phylogenetic noise generated by too many possible alignment errors, we took the conservative step of excluding from the analysis the most variable genes, and included only the four that are most conserved: *cox1*, *cox2*, *cox3*, and *cob*. The concatenated alignment of these amino acid sequences totalled 1413 positions.

Table 1. List of taxa used for the analysis, with indication of the GenBank accession number for the sequence of the mitochondrial genome

acc. no.	taxon	taxonomical assignment
NC_002735	*Tetrodontophora bielanensis*	HEXAPODA, Collembola
NC_005438	*Gomphiocephalus hodgsoni*	HEXAPODA, Collembola
to be submitted	*Japyx solifugus*	HEXAPODA, Diplura, Japygidae
NC_005437	*Tricholepidion gertschi*	INSECTA, Zygentoma
NC_001712	*Locusta migratoria*	INSECTA, Orthoptera
NC_002609	*Triatoma dimidiata*	INSECTA, Hemipteroid, Heteroptera
NC_003081	*Tribolium castaneum*	INSECTA, Endopterygota, Coleoptera, Tenebrionidae
NC_003372	*Crioceris duodecimpunctata*	INSECTA, Endopterygota, Coleoptera, Crysomelidae
NC_002084	*Anopheles gambiae*	INSECTA, Endopterygota, Diptera, Culicidae
NC_003368	*Ostrinia furnacalis*	INSECTA, Endopterygota, Lepidoptera, Pyralidae
NC_001620	*Artemia franciscana*	CRUSTACEA, Branchiopoda, Anostraca
NC_003058	*Pagurus longicarpus*	CRUSTACEA, Malacostraca, Decapoda, Anomura
NC_004251	*Panulirus japonicus*	CRUSTACEA, Malacostraca, Decapoda, Palinura
NC_003343	*Narceus annularus*	MYRIAPODA, Diplopoda
NC_003344	*Thyropygus* sp.	MYRIAPODA, Diplopoda
NC_003057	*Limulus polyphemus*	CHELICERATA, Merostomata, Xiphosura
NC_001636	*Katharina tunicata*	MOLLUSCA

In order to minimize systematic errors due to unequal base composition and uneven rates of evolution across sequences (Swofford et al. 1996), and to reduce the data set to manageable size, we excluded taxa when they failed tests for biased base composition and rates of evolution using the approach described in Nardi et al. (2003), as well as those taxa which were phylogenetically too close, as they would not add any significant information. A total of 17 taxa (Table 1) were subjected to phylogenetic analysis.
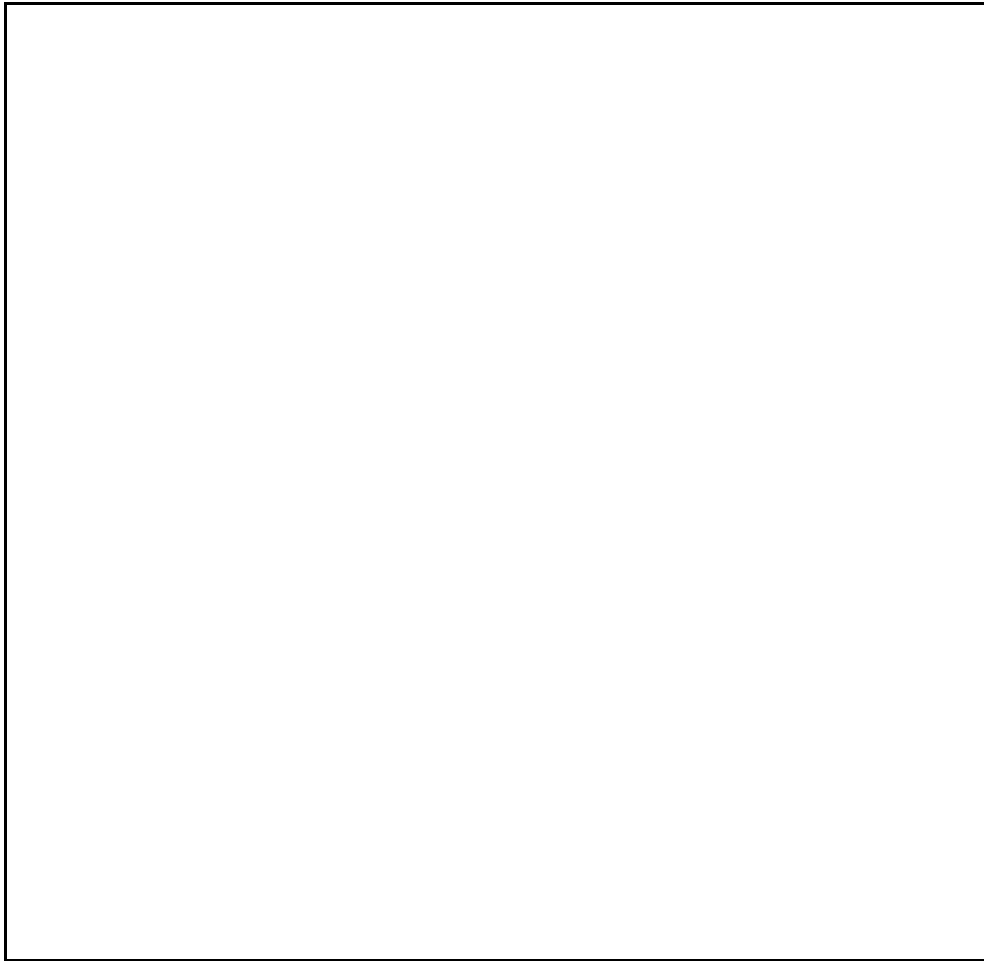
Figure 1. Phylogenetic tree obtained on the concatenated amino acid sequences of the four genes using a Bayesian Inference approach (MrBayes, 500,000 generations, burn in=150,000) with a GTR + invgamma model of evolution. Numbers at the nodes indicate posterior probabilities.

We performed a Bayesian analysis using MrBayes (Huelsenbeck & Ronquist 2001), with the GTR + invgamma model of evolution, to produce the tree shown in Fig. 1. The Bayesian approach has a considerable computational advantage over Maximum Likelihood methods for analysing large data set (Huelsenbeck et al. 2001), and it allows the use of a very complex but more realistic model of evolution such as the GTR + invgamma. The tree is rooted by specifying the mollusk *Katharina tunicata* (Boore & Brown 1994) as an outgroup. This tree supports, with high posterior probabilities, the taxon Pancrustacea, and the monophyly of Insecta, but does not support monophyly of Hexapoda. Both the collembolans and the dipluran *J. solifugus*, in fact, are placed at the base of a clade joining all Insecta with the three crustaceans. The same analysis (MrBayes, with GTR + invgamma,

with 500,000 generations, burn in=150,000) was also run on each of the four genes separately. While the *cob* gene gave the same results as the concatenated data set, with highly supported Pancrustacea and Insecta, but no support for Hexapoda, neither of the other three genes recovered these groups with high confidence. The better performance of the analysis of the concatenated data set over the analysis of single genes may be due to the fact that it averages the discrepancies across genes due to different selective pressures and functional constraints. This was shown earlier for mitochondrial genes by Cao et al. (1994).
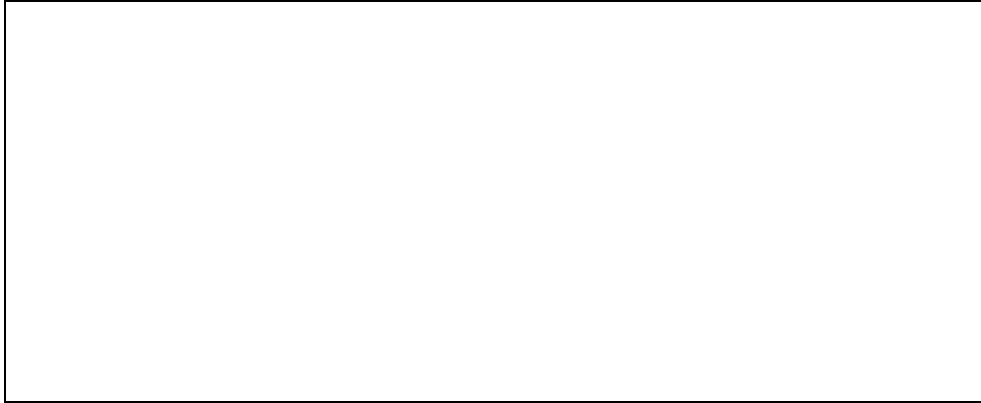

Figure 2. Test of significance of the difference in likelihood scores (mtREV24 + Γ model) among seven selected topologies depicting different potential relationships of the crucial taxa. pKH: Kishino-Hasegawa test (Kishino & Hasegawa 1989). pSH: Shimodaira-Hasegawa test (Shimodaira 2002). COL: Collembola (*G.hogdsoni* & *T.bielanensis*); DIP: Diplura (*J.solifugus*); INS: Insecta; CRU: Crustacea (*A.franciscana*, *P.longicarpus* & *P.japonicus*).

We evaluated statistically the relative likelihood of seven possible relationships for the most crucial taxa using PAML (Yang 1997) with the mitochondrially-based mt-REV24 + Γ model of evolution (Fig. 2). Although this model was developed for mammalian mitochondrial genes, and was found to lack wide generality (Liò & Goldman 2002), it is still one of the best available models to study phylogenetic relationships using amino acid sequences of mitochondrially-encoded proteins. For this analysis, we assumed monophyly for each of Collembola, Diplura, Insecta, and Crustacea, and considered the relationships among these groups. Five of the seven topologies imply non-monophyly of Hexapoda, including the one that has the best score (#3 in Fig. 2) which shows a clade uniting Collembola with Diplura and a clade uniting Insecta with Crustacea. The topologies assuming monophyly of Hexapoda (#1 and #2 in Fig. 2) have significantly lower scores with both the Kishino-Hasegawa (KH: Kishino & Hasegawa 1989) and the Shimodaira-Hasegawa (SH: Shimodaira 2002) tests, except for the topology #2 with the SH test. We also used PAML (and the mtREV24+Γ model) to test the seven different topologies using each the four genes individually. Fig. 3 shows the best topologies selected in each analysis, and their significance; as in the analysis shown before with GTR + invgamma model and MrBayes, *cox1* gives the same results as the concatenated data set (hexapod non-monophyly, with both Collembola and Diplura outside the Hexapoda, significantly better than hexapod monophyly), while the remaining three genes did not provide significant resolution to distinguish the two hypotheses.
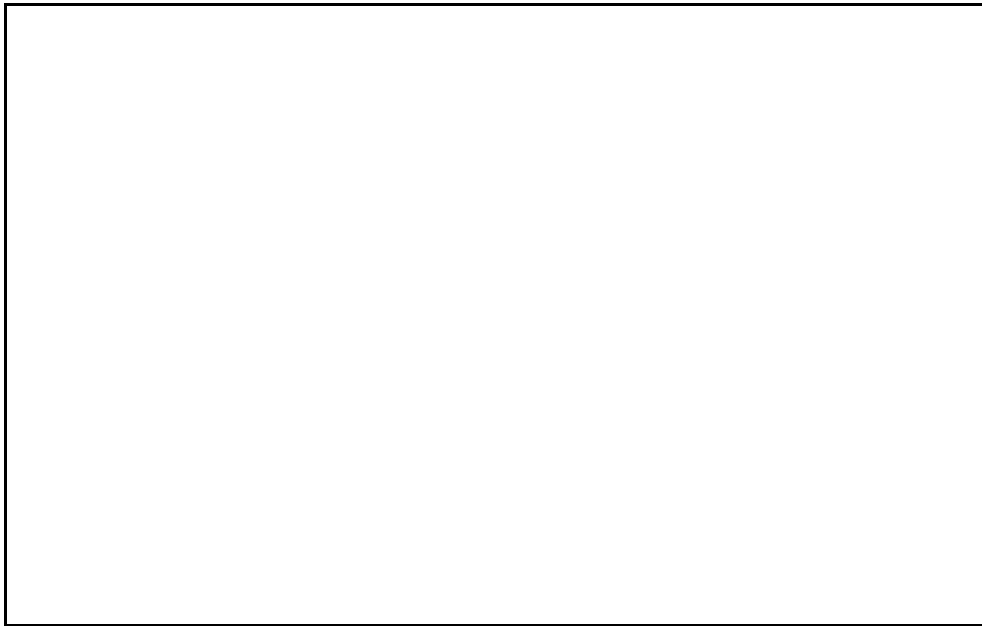
Figure 3. Level of significance of different tree topologies selected with the mtREV24 + Γ model in each single gene, and in the concatenated data set.

4   EVOLUTIONARY IMPLICATIONS OF HEXAPOD NON-MONOPHYLY

The data shown here from the analysis of mitochondrial genomes collectively support non-monophyly of Hexapoda as traditionally defined, with both Collembola and Diplura placed outside the Hexapoda, and with Crustacea (or, perhaps, just some crustacean groups) as the sister-taxon of Insecta. Collembolans and diplurans seem to have branched off very early from their pancrustacean ancestor, either before all present crustaceans differentiated from ectognathan insects, or as a derived group of one or more lineages of crustaceans. Our data, and particularly the lack of a denser sampling of crustacean lineages, do not allow us to resolve whether crustaceans are mono- or paraphyletic, and while the tree in Fig. 1 clusters the three crustacean taxa in a single clade (as in Negrisolo et al. 2004), the analysis of the larger data set, and also other molecular studies based on mitochondrial genes (Wilson et al. 2001; Nardi et al. 2001, 2003), suggest that some crustacean lineages (namely the Malacostraca) might be more closely related to insects than any other crustacean. The hypothesis of paraphyly of Crustacea with respect to insects is also suggested by other molecular studies (Regier & Shultz 1997, 2001; Garcia-Machado et al. 1999; Shultz & Regier 2000), and corroborated by other lines of evidence (Schram & Jenner 2001; Fanebruck et al. 2004).

The hypothesis of hexapod non-monophyly implies that the features which were typically claimed to support the taxon Hexapoda, such as, for instance, terrestrialization, body tagmosis, and a three-legged habitus, have independently evolved in different lineages of pancrustacean arthropods. It is well known that terrestrialization has occurred

independently several times among different lineages of arthropods, and it should come as no surprise if one (or more) additional independent terrestialization event is discovered among apterygotans (Negrisolo et al. 2004). Furthermore, the traditional association between terrestrialization and the tagmosis pattern (and the six-legged habitus) was challenged by the recent discovery of a presumed marine hexapod (Haas et al. 2003), with six legs and a tagmosis pattern intermediate between those of insects and crustaceans. Entognathan apterygotes also show a variety of peculiar characters with respect to "true insects", for example: entognathous mouthparts, different numbers of abdominal segments (12 in proturans, six in collembolans), the absence of antennae in proturans, and the absence of accessory microtubules in the sperm of proturans and collembolans (Dallai & Afzelius 1999). Although some of these characters might well be autapomorphic or symplesiomorphic with respect to Hexapoda (Kristensen 1981), and phylogenetic analysis of such morphological characters is difficult at such a deep level of divergence, indeed monophyly of Hexapoda is not well supported by either molecular (Friedrich & Tautz 2001) and morphological (Bitsch & Bitsch 1998; Klass & Kristensen 2001) data.

In the scenario of non-monophyletic hexapods, alternative hypotheses may be considered for the phylogenetic relationships of Collembola and Diplura (leaving aside Protura, for which no molecular data are yet available). The monophyly of Collembola seems apparent and supported by all molecular analyses (Fig. 1). Monophyly of Diplura has been questioned on the basis of the structure of ovarioles (Stys & Bilinski 1990) and other morphological characters, but there seems to be enough evidence (Bitsch & Bitsch 1998) to reject the monophyly of Entognatha (Protura+Collembola+Diplura). The placement of Diplura as the sister taxon of Ectognatha (=Insecta) is supported also by the presence of accessory tubules to the sperm axoneme, a putative synapomorphic feature of Insecta (Dallai & Afzelius 1999), but in contrast with our phylogenetic analysis which places Diplura, as well as Collembola, outside the Hexapoda.

5 HOW IS IT LIKELY THAT NEW DATA WOULD SOLVE THE CURRENT-DAY PHYLOGENETIC ISSUES?

The scientist to whom this special issue is dedicated, concluded his discussion concerning the Atelocerata-Pancrustacea controversy by pointing out that the two taxa were *"alternative hypotheses to be explored in the light of more information yet to be gathered"* (Schram & Jenner 2001). The same words could certainly be used for describing the controversy between monophyly *vs.* paraphyly of Hexapoda. So far, although the evidence available from mitochondrial genomics is not conclusive, the data at hand suggest that the hypothesis of hexapod non-monophyly deserves consideration and further scrutiny.

In order to complete the overall picture, new taxa need to be included in these analyses (i.e. additional mitochondrial genomes need to be sequenced), namely proturans, campodeid diplurans, and, among ectognathans, microcoryphians and other zygentomans. At the same time, methods of phylogenetic analysis are improving, following the exponential amount of molecular data gathered, and, concerning mitochondrial proteins, considerable improvements have been made by using models of evolution which take structural information into account (Liò & Goldman 2002). Finally, Bayesian inference more efficiently allows the combination of molecular and morphological data, and the

incorporation into the phylogenetic analysis of prior information based on morphological characters. All these steps, more taxa, better methods, and more efficient combination of information will certainly help in reconstructing this fundamental step in the evolution of arthropods.

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